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CITATION:

Watanabe, Katsutoshi ...[et al]. Comparative phylogeography of diadromous and freshwater daces of the genus *Tribolodon* (Cyprinidae). *Ichthyological Research* 2018, 65: 383-397

ISSUE DATE:

2018-07

URL:

<http://hdl.handle.net/2433/250233>

RIGHT:

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# Comparative phylogeography of diadromous and freshwater daces of the genus *Tribolodon* (Cyprinidae)

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Suggested running head: Comparative phylogeography of *Tribolodon*

Type of paper: full paper

Number of text pages: 27; Number of figures: 8; Number of tables: 1; Number of ESM: 4 (3 Figs. and 1 Table)

## Abstract

Far Eastern daces, genus *Tribolodon* (Cyprinidae), are thought to have diversified and developed unique diadromous life histories under changing conditions in the Sea of Japan and the surrounding environment. To examine the relationships between life history traits, distribution, and genetic population structures, we conducted a comparative phylogeographic analysis using partial mtDNA sequence data from samples collected over almost the full ranges of all four *Tribolodon* species. Phylogenetic analyses revealed several intraspecific haplotype groups that differentiated in the early Pleistocene to the Pliocene with or without geographic overlaps. A time-calibrated phylogeny suggested that the relatively smaller geographic ranges of the strictly freshwater species, *T. sachalinensis* and *T. nakamurai*, were explained not by the recent origins of these species, but by their limited dispersal abilities and smaller historical population sizes. The wider-ranging diadromous species, *T. brandtii* and *T. hakonensis*, exhibited similar major phylogeographic structures in their distributions, but the chronological order and timing of formation of this structure largely differed between the two species. In addition to those differences, the overlapping patterns of the differentiated intraspecific lineages in these species suggest dynamic, but somewhat restricted dispersal during the Plio-Pleistocene. *Tribolodon hakonensis*, one of the most widespread species of East Asian freshwater fishes, included both common and unique phylogeographic patterns compared to other fish species; the unique patterns (i.e., its wide range across freshwater biogeographic boundaries like the sea and mountains) would reflect its ecological features as a remarkable generalist inhabiting lakes, upper and lower reaches of rivers, and even coastal areas.

**Keywords** Freshwater fish · Mitochondrial DNA (mtDNA) · Far East Asia · Central Highland · Diadromy

## Introduction

The composition and regional heterogeneity of biological communities are determined through range expansion, geographical differentiation, and local extinction of component species (e.g., MacDonald 2003; Emerson and Gillespie 2008). These distribution processes are linked to the geological and climatic history of the regions where the communities occur. The processes are affected by both extrinsic factors (such as barriers or filtering, either by physiochemical environments or biological interactions) and intrinsic factors (e.g., ecological and life history traits, such as dispersal ability). Among the distribution processes, range expansion is the primary biogeographical process by which new species can become potential members of a community. The extent of range expansion would reflect a species' dispersal ability, as well as the length of time over which the species has dispersed. Intraspecific population structure, or phylogeographic information, is crucial for inferring historical range expansion (Avice 2000; Templeton 2006). Comparing phylogeographic patterns among component species with various ecological traits can deepen our understanding of the basic framework of community assembly processes on a regional scale.

Freshwater fish are a suitable group for studying the relationships between geological/climatic changes and the distribution of organisms, because the dispersal ability of these species is fundamentally restricted to freshwater systems that have been affected by such changes (Avice et al. 1987; Watanabe et al. 2016). This group includes strictly freshwater species (which are intolerant to salt water), diadromous species (which migrate between freshwater and marine environments), and peripheral freshwater species (which include brackish and sporadic freshwater fish) (see Myers 1949; Berra 2001). Different life history modes and their evolution can affect the distribution range and population structures of freshwater species groups. Specifically, the ranges of diadromous and peripheral species are likely to expand more quickly than those of strictly freshwater species, because the former groups could potentially disperse through the sea. Such dispersal often facilitates greater gene



flow among local populations, which in turn inhibits population differentiation (Aulsebrook 2000; McDowall 2001, 2008). On the other hand, diadromous species often give rise to landlocked, regionally isolated populations and even species (e.g., charrs, sticklebacks; Yamamoto et al. 2004; Takahashi et al. 2016). The relative importance of these two aspects may depend on specific features of migratory ecology (e.g., life history phase and timing of migration) as well as geological and climatic conditions. Comparisons of phylogeographic patterns among closely related species with different life history modes may help to clarify the relationships between ecological traits and distribution patterns, by eliminating extraneous factors due to phylogenetic constraints.

The Far Eastern dace genus *Tribolodon* is a unique group within the large family Cyprinidae, encompassing a variety of life histories, including fluvial (freshwater residential), lacustrine (migratory between river and lake), and diadromous (anadromous or amphidromous; sea run) modes (Nakamura 1969). The variability of life histories in *Tribolodon* makes it a good group for examining the relationship between distribution processes and ecological differences. This genus consists of four species, distributed around the Sea of Japan, and its evolution is likely to have been shaped by the formation of the Sea of Japan since the Miocene (Nishimura 1974; Sakai 1995; Sakai et al. 2002; Imoto et al. 2013). The genus includes two diadromous species (*Tribolodon brandtii* and *T. hakonensis*) and two freshwater resident species (*T. nakamurai* and *T. sachalinensis*), whose geographic range sizes are various (Fig. 1; see “Materials and methods” for more details). The four species are morphologically similar to each other, and interspecific hybrids frequently occur in sympatric areas (Sakai and Hamada 1985; Sakai 1995; Atsumi et al. 2017). Because of these features, *Tribolodon* species have attracted considerable attention in ecological, physiological, and evolutionary contexts, particularly in connection with the geological history of the Sea of Japan (e.g., Sakai 1995; Kaneko et al. 1999; Sakai et al. 2002). The speciation and distribution patterns of these species, along with related phoxinini groups, have been repeatedly examined using allozyme and mitochondrial DNA (mtDNA) data (Sakai et al. 2002, 2006; Sasaki et al.

2007; Ryazanova and Polyakova 2012; Brykov et al. 2013; Imoto et al. 2013). However, to date, no studies have compared the detailed phylogeographic patterns of these species with full geographic sampling using high-resolution genetic markers.

In this study, we analyzed the phylogeographic patterns of all four *Tribolodon* species using mtDNA sequence data from samples collected over almost the full ranges of these species. Comparing patterns among these four species in a common spatiotemporal framework, we explored the following questions: whether the wideness of each of their ranges is attributable to life history modes or the timing of the origin of the species; whether some *Tribolodon* species show common phylogeographic patterns; and, if so, whether these patterns were formed on the same timescale. Finally, we discuss the evolutionary and distribution history of *Tribolodon* species compared to those of other freshwater fishes distributed around the Sea of Japan.

## Materials and methods

*Background of study area and species.* The four *Tribolodon* species are distributed around the Sea of Japan, including the Japanese Archipelago, Sakhalin Island, the southern Kuril Islands, the Russian Maritime Territory (Primorsky Region), and the Korean Peninsula (Fig. 1). The Sea of Japan began to open at the eastern margin of the Eurasian continent in the Early Miocene (Tada 1994; Yonekura et al. 2001). The sea has subsequently experienced drastic environmental fluctuations, including changes in temperature, salinity, and oxygen concentration, due to periodic closing of the southern channel of the sea, blocking the inflow of the Tsushima Current from the southwest (e.g., Jolivet et al. 1994; Tada 1994; Kitamura and Kimoto 2006). The Japanese Archipelago is the center of distribution of *Tribolodon*; all four species of the genus can be found in this area. Strictly freshwater fish native to the Japanese Archipelago show primary differences across the boundaries of the Ishikari Plain in

western Hokkaido and the Fossa Magna region (Central Highlands area) in central Honshu (Fig. 2; e.g., Watanabe 2012; Watanabe et al. 2016).

The genus *Tribolodon* (Far Eastern dace, or red fin) belongs to subfamily Leuciscinae in the family Cyprinidae. *Tribolodon* is closely related to the genus *Pseudaspius*, whose sole species, *Pseudaspius leptcephalus*, occurs in the basins of the Amur River, Sakhalin Island, and Mongolia (Sakai et al. 2002; Sasaki et al. 2007; Imoto et al. 2013). Some studies have suggested that *Tribolodon* is paraphyletic, with a closer relationship between *P. leptcephalus* and one or more species of *Tribolodon* than between *Tribolodon* species (Sasaki et al. 2007; Imoto et al. 2013). The *Tribolodon*–*Pseudaspius* clade is included in the Far Eastern phoxinin group together with *Rhynchocypris* (the Far Eastern “*Phoxinus*”; Sakai et al. 2006; Imoto et al. 2013).

One of the four valid species of the genus, *Tribolodon hakonensis* (Japanese common name, “Ugui”), exhibits intraspecific variation in life history, including fluvial, lacustrine, and sea-run types. This species occurs in rivers, lakes, and river mouths to coastal areas throughout the range of the genus, and possesses high osmoregulatory capability (Fig. 1; Sakai 1995). It is one of the most widespread freshwater fish species in East Asia. The distributions of the other three species, however, are relatively restricted to the northern parts of the range of the genus (Fig. 1). *Tribolodon brandtii* has an anadromous lifestyle, and includes two subspecies: *Tribolodon brandtii brandtii* (“Jusan-ugui”) and *Tribolodon brandtii maruta* (“Maruta”) (Sakai and Amano 2014). The remaining two species, *Tribolodon sachalinensis* (“Ezougui”) and *Tribolodon nakamurai* (“Ukekuchi-ugui”), are freshwater residents (Sakai 1995; Sakai and Imai 2002; Imai et al. 2008). Some authors recognize a fifth species, *Tribolodon* sp., from the Primorsky region (e.g., Semina et al. 2006; Brykov et al. 2013), which probably corresponds to one of the regional groups of *T. hakonensis* in the present paper (see “Discussion”).

*Specimens.* In total, 400 specimens of the four *Tribolodon* species were collected from 49 river systems in Japan (locality code 1–43), Sakhalin (44–45) and Primorsky (46–47) in

Russia, and the Korean Peninsula (48–49) between 1991 and 2016 (Fig. 2; Table 1). Because of the small number of specimens of *T. nakamurai* ( $n = 3$ ), our analyses included four additional *T. nakamurai* sequences retrieved from a DNA database (AB198967, 198968, Sasaki et al. 2007; AB218896, Saitoh et al. 2006; KP219892, N. Polyakova, unpublished data). Specimens with suspected introgressed mtDNA, i.e., those possessing mtDNA of a different species from that inferred from a previous allozyme analysis (Sakai et al. 2002), were not included in the present study. A specimen of *Pseudaspius leptcephalus* from the Chita River (Amur River system, Chita, Russia) was also included in the analyses because of its close relationship to *Tribolodon* (Sakai et al. 2002; Sasaki et al. 2007; Imoto et al. 2013). An additional sequence (AP009058; Saitoh et al. 2006) from this species was also used. Four phoxinin species were used as outgroups: sequences were obtained from one specimen each of *Rhynchocypris lagowskii steindachneri* and *Rhynchocypris oxycephalus*, collected from the Ishida River, Lake Biwa system, Shiga, central Honshu, Japan, and previously published sequences for *Rhynchocypris percnurus* (AP009150; Imoto et al. 2013) and *Oreoleuciscus potanini* (AB626851; Imoto et al. 2013) were also included.

**DNA sequencing.** Total genomic DNA was isolated from a piece of fin or muscle preserved in 99 % ethanol using a standard phenol/chloroform method or Genomic DNA Purification kit (Promega, Tokyo, Japan). Polymerase chain reaction (PCR) amplification was carried out using the primer pair L14724 (5'- TGA CTT GAA RAA CCA YCG YYG -3') (Palumbi et al. 1991) and H15915 (5'- ACC TCC GAT CTY CGG ATT ACA AGA C -3') (Aoyama et al. 2000) to amplify the mtDNA cytochrome *b* gene region (*cytb*) for all specimens. PCR conditions consisted of 30 cycles of denaturation (94 °C for 15 s), annealing (48 °C for 15 s), and extension (72 °C for 60 s) on a thermal cycler (ASTEC, Fukuoka, Japan). Then, PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, OH) or Illustra ExoStar (GE Healthcare Japan, Tokyo, Japan) at 37 °C, and sequenced on an automated DNA sequencer (GA3130xl; Applied Biosystems, Foster City, CA) with primer H15915 to obtain the 3'-half sequences of *cytb* (758 bp; hereafter, “short sequences”), using a BigDye

Terminator Cycle sequencing FS Ready Reaction kit ver. 3.1 (Applied Biosystems). We obtained longer sequences (2,941 bp; “long sequences”) of selected samples ( $n = 39$ ) with haplotypes representing each major clade, as detected in the analysis of the short sequences. These long sequences included parts of the *cytb* (1,084 bp), cytochrome *c* oxidase subunit I (COI; 655 bp), and 16S ribosomal RNA (16S; 1,202 bp) regions, and allowed us to construct a more credible phylogeny. We used the following primers: FishCO1F (5'- TCA ACC AAC CAC AAA GAC ATT GGC AC -3') and FishCO1R (5'- TAG ACT TCT GGG TGG CCA AAG AAT CA -3') for COI (annealing 52 °C) (Ward et al. 2005), and L1854 (5'- AAA CCT CGT ACC TTT TGC AT -3'; Watanabe et al. 2000) and H3058 (5'- TCC GGT CTG AAC TCA GAT CAC GTA -3'; Kitaura et al. 1998) for 16S (annealing 52 °C). The resulting sequences were deposited in the DNA databank DDBJ/EMBL/GenBank (accession numbers LC277189–LC277704). The haplotype frequencies of each population were deposited in the Genetic Diversity and Distribution Map (GEDIMAP) freshwater fish database (<http://gedimap.zool.kyoto-u.ac.jp>; Watanabe et al. 2010) with population IDs P2201–2260.

*Phylogenetic and phylogeographic analyses.* DNA sequences were edited and aligned with ClustalW and by eye using MEGA 7.0 (Kumar et al. 2016). Phylogenetic analyses were conducted on both datasets (short and long sequences) using maximum likelihood (ML) and Bayesian inference (BI) methods. The ML tree and 1,000 non-parametric bootstrap replicates (ML BP) were obtained under the GTRCAT model (partitioning by gene in the long-sequence dataset) implemented in RAxML ver. 8.0 (Stamatakis 2014).

In BI analyses, we estimated the time-calibrated tree based on the long sequence data with the prior assumptions for molecular substitution rate and date of the geological barrier for population isolation, calculated according to the relaxed molecular clock model (Drummond et al. 2006). Substitution and clock model parameters were estimated separately for each of the three genes. We used molecular substitution rates estimated for teleost *cytb*, ranging from roughly 0.3 % to 1.5 % per million years (Myr) per lineage (e.g., Burrridge et al. 2008; Watanabe and Takahashi 2010) with a mean of 0.76 % (for European cyprinids;

Zardoya and Doadrio 1999). We adopted the uncorrelated lognormal relaxed clock model with a normal prior distribution for mean molecular substitution rate, giving 0.76 % (SD = 0.5 %) for *cytb*; this is a lax constraint, covering <0.3–1.6 % in a 95 % interval. The mean substitution rates of the other two genes (COI and 16S) were estimated using the CTMC rate reference prior (Ferreira and Suchard 2008). The uplift of the Suzuka Mountains in central Honshu Island 1.0–1.5 million years ago (Mya) (Fig. 2; Yokoyama 1988; Kawabe 1994) was hypothesized to have worked for the relevant divergence and used for the corresponding node age in *T. hakonensis* as a geological constraint (see “Results”). This constraint was specified as a lognormal prior distribution, ranging from approximately 1.1 to 1.5 Mya in the 95 % range [mean = 1.3 Mya; log(SD) = 0.1; offset = 0]. To examine the concordance between molecular substitution rates and geological constraints, we also constructed a time tree using only the calibration by molecular substitution rate as a more lax constraint.

The Bayesian phylogenetic tree and divergence time (the time of the most recent common ancestor; tMRCA) were estimated using the above assumptions and a Yule (speciation) tree prior with BEAST 1.8.4 (Drummond and Rambaut 2007). The substitution models were selected using the Bayesian information criterion (BIC) in jModelTest 2.1.6 (number of substitution schemes = 5; Darriba et al. 2012): TrN+G for *cytb* and COI and HKY+G+ I for 16S. All other model parameters were set to the default priors. For MCMC analyses, we performed two independent runs of 50 million generations to confirm the consistency of the results. We sampled every 2,000 generations and removed the first 10 % of samples as burn-in. The convergence of the chains to the stationary distribution and large effective sample size (>200) were confirmed using Tracer v.1.6 (Rambaut et al. 2013). The consensus tree with median node heights was calculated by TreeAnnotator v.1.8.4 in the BEAST package, and the tree was visualized using FigTree v.1.4.3 (Rambaut 2016). Node support was evaluated based on posterior probability (BI PP).

A statistical parsimony network for each species was constructed using TCS v1.2.1 (Clement et al. 2000) at a 95 % confidence limit for the mtDNA haplotypes obtained. Loops

were resolved so that the topology becomes consistent with that of the ML tree. The grouping of haplotypes followed the nesting rule by Templeton and Sing (1993).

## Results

**Major divergences and their timing.** The phylogenetic tree based on the “short sequences” supported the monophyly of each species and subspecies of *Tribolodon* with high bootstrap values (89–100 %), with the exception of *T. brandtii*, whose monophyly was not supported (Fig. 3). The monophyly of *T. brandtii* + *T. nakamurai* + *T. sachalinensis* + *Pseudaspius leptocephalus* was also supported with a high bootstrap value (94 %), but this result was less well supported in ML and BI trees based on the “long sequences” [ML BP = 77 %, BI PP = 0.84; Fig. 4, Electronic supplementary material (ESM) Fig. S1].

In the time-calibrated tree based on the long sequences with the prior assumptions for the *cytb* substitution rate and the date of the Suzuka Mountains vicariance (see below), the mean evolutionary rates (nucleotide substitutions per million years per lineage) of the respective genes were estimated at a median of 0.79 % [95 % highest probability density (HPD), 0.58–1.1 %] for *cytb*, 0.48 % (0.32–0.67 %) for COI, and 0.22 % (0.15–0.31 %) for 16S, and no remarkable changes appeared over the tree. When only the molecular substitution rate was used, without the Suzuka Mountains vicariance calibration, similar, slightly faster evolutionary rates (1.02 % for *cytb*, 0.62 % for COI, and 0.29 % for 16S) were obtained. The time of isolation by the Suzuka Mountains was consequently estimated to be slightly more recent than that with the geological constraint [1.26 (1.03–1.52) vs. 0.96 (0.35–2.53) Myr; see below], but the 95 % HPDs were completely overlapped. We thus concluded that there was no remarkable discrepancy between the evolutionary rate and geological constraints assumed in our analyses.

In the time-calibrated tree, the median tMRCA of *Tribolodon* and *Pseudaspius* species



(node 1 in Fig. 4) was estimated to be 8.66 Myr (95 % HPD, 5.83–11.79). Interspecific relationships in this tree were not well resolved, except for the close relationship between *T. brandtii* and *T. nakamurai*. However, the time tree and haplotype networks revealed clear intraspecific groups of *Tribolodon* species, associated with geographical distribution.

**Phylogeography of *T. brandtii*, *T. nakamurai*, and *T. sachalinensis*.** *Tribolodon brandtii* was clearly divided into two haplotype groups (ML BP = 100 %, BI PP = 1.0 for both) with an average sequence difference of 3.9 % ( $\pm 0.2$  SD) in the short sequences (ESM Table S1). These groups correspond to the subspecies *T. brandtii brandtii* and *T. brandtii maruta* (TBB and TBM; Figs. 4, 5), which are distributed around the Sea of Japan and on the Pacific side of eastern Honshu, respectively (Fig. 5). The tMRCA between these two subspecies was estimated at 3.04 Myr (95 % HPD, 2.00–4.29 Myr), which was the greatest amount of intraspecific divergence among *Tribolodon* species (node 3 in Fig. 4). *Tribolodon brandtii brandtii* was further separated into two haplotype groups (TBB1 and TBB2) with an average sequence difference of 1.6 % ( $\pm 0.2$  SD). These groups were distributed in the northern (northwestern Honshu, Hokkaido, and Sakhalin) and southwestern parts (Korea and southern Primorsky) around the Sea of Japan, respectively (Fig. 5). The boundary of these two groups was probably located in central to northern Primorsky, but due to insufficient sampling, its location was not clear. The divergence time between the two groups was estimated at 0.95 Myr (0.52–1.47 Myr; node 4). Both groups include widespread haplotypes such as bb06 and 08 (TBB1; locality code 14-Shinano River to 44-Sakhalin) and bb10 and 11 (TBB2; 47-Peter the Great Bay to 49-Korea) (Table 1).

*Tribolodon nakamurai* formed a monophyletic group with *T. brandtii*; the tMRCA of the two species was estimated at 3.29 Myr (2.17–4.62 Myr; node 2 in Fig. 4). Three haplotypes (n01–03) were identified. No geographical structure was found in the distribution of this species, although the sample size was too small to detect it, if present (Fig. 6; Table 1).

The estimated tMRCA of *Tribolodon sachalinensis* was 0.66 Myr (0.36–1.07 Myr; node 5 in Fig. 4), and there were no clear genetic divisions within the species. However, some



haplotypes, such as sa10 and 17, showed differentiation from major haplotypes (Fig. 7).

**Phylogeography of *T. hakonensis*.** *Tribolodon hakonensis* showed high intraspecific diversity, including six mtDNA haplotype groups (TH1–TH6) with a tMRCA of 2.61 Myr (1.72–3.59 Myr; node 6 in Fig. 4). The distributions of these groups partially overlapped (Fig. 8). The group TH6 was the first to be derived (ML BP = 95 %; BI PP = 1.0) and was distinct from the other groups (TH1–TH5) in a statistical parsimony network at 95 % or even 90 % confidence limits. The average sequence difference between TH6 and TH1–TH5 haplotypes was 3.2 % ( $\pm 0.3$  SD). Similar to TBB2 of *T. b. brandtii*, TH6 was found in the southwestern surroundings of the Sea of Japan (Korea and southern Primorsky); however, a subgroup (TH6-2, the derived group as inferred from root position) extended into Tsushima Island and the northern part of Kyushu, Japan (loc. codes 43 and 40; Figs. 2, 8d). The divergence time between TH6-1 (continental) and TH6-2 (Japan) was estimated at 0.54 Myr (0.25–0.89 Myr; node 11 in Fig. 4).

The Suzuka Mountains separated the range of the group TH5 from the ranges of the other northeastern groups (TH1–TH4) (Fig. 8). Although the phylogenetic relationships among groups TH1–TH5 were not fully resolved, the sister relationship of TH5 and TH3 + TH4 was supported by a moderately high bootstrap value in the ML analysis (80 %; Fig. 4; ESM Fig. S1). Hence, the timing of uplift of the Suzuka Mountains was used to calibrate the tree (node 8 in Fig. 4; see “Materials and methods”), giving a tMRCA of 1.26 (1.03–1.52) Myr for TH5 + (TH3 + TH4). Diversification of TH1–TH5 was inferred to have started 1.30 (1.03–1.62) Mya (node 7 in Fig. 4).

TH1 was restricted to the northern part of the range of *T. hakonensis*, i.e., the Tumnin River (Khabarovsk), Sakhalin, and Hokkaido (Fig. 8a). TH2 was distributed on the Pacific side of eastern Honshu (Fig. 8a), with a range similar to that of *T. b. maruta* (TBM). TH3 was a major group in the middle to northern parts of the range (Fig. 8b), overlapping with the range of TH1 and part of the range of TH2. TH3 consisted of some major widespread haplotypes (e.g., h37 and h47; Table 1) with several close haplotypes.

TH4 was mainly distributed on the Pacific side of central Honshu, but was also found in the rivers flowing into the Sea of Japan in midto northern Honshu, across the Fossa Magna region (Figs. 2, 8c). The haplotypes in the latter area (TH4-2) were differentiated from those on the Pacific side (TH4-1), but these subgroups were sympatric in the intermediate mountainous locations (loc. codes 24, 26). The tip position of TH4-2 in the haplotype network suggested the dispersal direction from the Pacific side to the Sea of Japan side (Fig. 8c). The divergence time between TH4-1 and TH4-2 was estimated at 0.43 Myr (0.25–0.65 Myr; node 9 in Fig. 4).

TH5 was the most diverse group. It was distributed in western Japan with a tMRCA of 0.56 Myr (0.34–0.80 Myr; node 10 in Fig. 4; Fig. 8d). Haplotypes h10–h12 were in a basal position in the haplotype network of this group (Fig. 8d, e; Table 1) and were only found in samples from the Lake Biwa system. In the Lake Biwa system, haplotypes of another subgroup (h33–35) were also found. TH5 contained at least five subgroups, which to some extent corresponded with the geographical distribution (Table 1; ESM Fig. S2).

**Comparisons of diversification patterns and timing.** The two diadromous *Tribolodon* species, *T. brandtii* and *T. hakonensis*, have wider ranges than the two freshwater species (Fig. 1), and estimates of their tMRCA were similar (ca. 3 Myr; Fig. 4). The divergence times of the two freshwater species, *T. nakamurai* and *T. sachalinensis*, were not necessarily shorter than those of the diadromous species, but the freshwater species had shorter tMRCA, equivalent to those of regional haplotype groups of the diadromous species (Figs. 3, 4). The two diadromous species showed similar phylogeographic patterns. That is, in both *T. brandtii* and *T. hakonensis*, distinct regional haplotype groups were found in the southwestern side of the Sea of Japan (Korea to Peter the Great Bay; TBB2 and TH6) and the Pacific side of eastern Honshu (TBM and TH2; Figs. 5, 8). However, the chronological order and timing of haplotype group divergence differed between the two species. In *T. brandtii*, the primary divergence (node 3 in Fig. 4) was between populations on the Pacific side (TBM) and the Sea of Japan side (TBB) (divergence time: 2.00–4.29 Myr of 95 % HPD, as mentioned above);

these two groups corresponded to the two different subspecies *T. b. maruta* and *T. b. brandtii* (see Sakai and Amano 2014). The second divergence occurred around the Primorsky region in this species (divergence time: 0.52–1.47 Myr; node 4 in Fig. 4). By contrast, the vicariance around the Primorsky region represents the primary divergence of *T. hakonensis*, with an estimated divergence time of 1.72–3.59 Myr (node 6 in Fig. 4). The haplotype group of *T. hakonensis* distributed in the Pacific side of Honshu (TH2) diverged more recently, around one million years ago (Fig. 4), and is partially sympatric with the TH3 group (Fig. 8).

The range of the TH3 haplotype group of *T. hakonensis* was similar to the range of *T. sachalinensis*, but the tMRCA was shorter in the former [0.23 (0.09–0.43) Myr for TH3 of *T. hakonensis* and 0.66 (0.36–1.07) Myr for *T. sachalinensis*; Fig. 4]. *Tribolodon sachalinensis* had a tMRCA rather similar to that of the clade TH3 + TH4 [0.71 (0.46–1.02) Myr].

## Discussion

**Comparative phylogeography of *Tribolodon* fishes.** Our time-calibrated mtDNA gene tree suggested that the wideness of a species' range depends more on life history modes than on the timing of the origin of the species. That is, the freshwater species (*Tribolodon nakamurai* and *Tribolodon sachalinensis*) are distributed over narrower ranges than the diadromous species (*Tribolodon brandtii* and *Tribolodon hakonensis*), although the former did not necessarily originate more recently. This conclusion, however, should be confirmed by information from nuclear genes, because it is based on the assumption that the mtDNA tree properly reflects the species history.

On the other hand, the tMRCAs were shorter in the freshwater species, suggesting their smaller historical population size compared with the diadromous species (e.g., Avise 2000). The implication is that geographic range and historical population size in *Tribolodon* species reflect their ecological features, particularly migration and range of tolerance for salinity and

temperature. Diadromy enabled by high osmoregulatory capability would have made it easier for the diadromous species to migrate to refugia and re-expand their range (McDowall 2001). Specifically, the variable life history mode and wide habitat tolerance (lower to upper reaches of rivers, lakes, and coastal areas) of *T. hakonensis* are likely to have played an important role in forming its remarkably wide distribution.

The two diadromous species, *T. brandtii* and *T. hakonensis*, had similar major phylogeographic structures, but their chronological order and timing of formation differed between these species. This indicates that their apparently similar phylogeographic patterns resulted from dispersal and isolation in different periods. The common patterns between the two species, i.e., the vicariance across the Primorsky region and the isolation of populations on the Pacific side of eastern Honshu, are partially supported by previous studies (Sakai et al. 2002; Sasaki et al. 2007), particularly those with large number of specimens from the Russian region (e.g., Brykov et al. 2011, 2013). The obvious geographic structure of these populations suggests that stepping-stone dispersal along the coast is not necessarily easy even for diadromous species; it is probably inhibited by long distances between river mouths, coastal topography, and the direction and intensity of ocean currents. Repeated opportunities for dispersal and isolation caused by environmental fluctuations around the Sea of Japan during the Plio-Pleistocene climatic oscillations (Tada 1994; Kitamura and Kimoto 2006) could have formed similar geographic patterns observed here in historically different ways.

Brykov et al. (2013) suggested that species in the Russian regions followed a common intraspecific divergence pattern; this assertion is partially supported by our results, which are based on wider geographic sampling. On the basis of mtDNA RFLP data, Brykov et al. (2013) found two or three mtDNA lineages that occur partially or largely sympatrically from Peter the Great Bay to Sakhalin Island in *T. brandtii brandtii*, *T. sachalinensis*, and *T. hakonensis* (excluding our TH6) (ESM Fig. S3). They estimated the intraspecific divergence (pairwise sequence difference) for these three species at 2.3 %, 2.9 %, and 2.1 %, respectively, and claimed that the intraspecific lineages diverged as a result of common historical factors for

isolation and secondary contact. Although it is difficult to directly compare our sequence data with their RFLP data, the distribution patterns and degrees of divergence suggest that our TBB1 and TBB2 of *T. b. brandtii* correspond to phylogroups 3 and 2 of Brykov et al. (2013). Haplotype sa17 and others probably correspond to their phylogroups 2 and 1 of *T. sachalinensis*, and TH1 and TH3 correspond to phylogroups 1 and 2 (or vice versa) of *T. hakonensis*. The pairwise uncorrected p-distances of our *cytb* data were 1.61 % for the TBB1 and TBB2 groups of *T. b. brandtii*, 1.03 % for Haplotype sa17 and other *T. sachalinensis* sequences, and 1.64 % for the TH1 and TH3 groups of *T. hakonensis* (ESM Table S1). These values support the conclusions of Brykov et al. (2013), at least with regard to *T. b. brandtii* and *T. hakonensis*, although it is more difficult to draw conclusions about *T. sachalinensis* due to the small number of Russian specimens in the present study. Furthermore, Brykov et al. (2013) showed the existence of the third group (phylogroup 1) of *T. b. brandtii*, which was distributed mainly around Peter the Great Bay; we may not have analyzed any samples of this group. The range of this group extends to Sakhalin Island (haplotype C of Brykov et al. 2013). This pattern suggests that partial secondary contact occurred on that island, involving *T. b. brandtii* immigrants that crossed the Primorsky vicariance.

**Phylogeography of *Tribolodon hakonensis*.** As a species with a remarkably wide distribution, *T. hakonensis* shares several common phylogeographic patterns with other freshwater fishes in Japan and adjacent regions. For example, *T. hakonensis* in the Japanese Archipelago is largely divided by the Suzuka Mountains (haplotype group TH3–TH4 vs. TH5); this mountain range, which was uplifted about 1.0–1.5 Mya (Yokoyama 1988; Kawabe 1994), is an important biogeographic boundary dividing species and populations of many other freshwater fishes (Watanabe et al. 2016). In addition, *T. hakonensis* populations west of the mountains have maintained diverse haplotypes, and different mtDNA lineages co-occur in some localities, as typified by the population in Lake Biwa, an ancient lake (Table 1; ESM Fig. S2). This phylogeographic pattern is observed in several widespread species in western Japan (Tabata et al. 2016).

The inferred penetration of the haplotype group TH6 from the continental regions (Primorsky to the Korean Peninsula) to Tsushima Island and even into a part of Kyushu Island in Japan (Fig. 8d) provides a good example of the colonization of western Japan by freshwater fish from the Korean Peninsula (Aoyagi 1957; Nishimura 1974; Mizuno 1987). This is also supported by allozyme data, which show that the continental allele \*85 at Prot-2\* can be found in Tsushima Island populations (Sakai et al. 2002). Our results, however, do not support the hypothesis that such colonization by continental freshwater fish occurred in the last glacial period (0.01–0.07 Myr ago) (e.g., Lindberg 1972; Nishimura 1974). Instead, genetic differentiation within TH6 suggests that colonization of western Japan occurred in the middle Pleistocene (0.25–0.93 Myr ago), even in the case of diadromous species. In recent Russian references, the southwestern continental population, most likely the same as our TH6 group, is treated as “*Tribolodon* sp.” (e.g., Semina et al. 2006; Brykov et al. 2011, 2013). In our data, the genetic differentiation between TH6 and TH1–TH5 (3.2 % uncorrected p-distance) is equivalent to that between the two subspecies of *T. brandtii* (3.9 %). Gudkov et al. (2010) reported morphological differences between southern Primorsky and Sakhalin populations. Further, Polyakova et al. (2015) stated the possibility that the southwestern continental population (*Tribolodon* sp.) was formed as a result of homoploid hybridization between *T. hakonensis* and *T. brandtii*, based on the data from four nuclear DNA loci. Inclusive morphological comparisons and genome-wide studies are necessary to determine the taxonomic and evolutionary status of the southwestern continental population of *T. hakonensis*.

Several aspects of the phylogeographic patterns of *T. hakonensis* are quite different from those of other freshwater fishes. Most strictly freshwater fish species are found only on one side of the Ishikari Plain in western Hokkaido; few fish species in western Japan occur north of the plain (Nishimura 1974; Watanabe 2012). The remarkably wide distribution of *T. hakonensis* across this boundary would be achieved by its diadromous, euryhaline, and eurythermal habits. On the other hand, multiple well-differentiated mtDNA haplotype groups

(e.g., TH1 and TH3) co-occur in the northern part of this species' range. This suggests repeated reduction and reexpansion of its distribution, involving genetic isolation and secondary contact, probably influenced by the global climate oscillation in the Pleistocene. A similar overlapping pattern of mtDNA haplotype groups is observed in the white-spotted char, *Salvelinus leucomaenis*, which is also a euryhaline fish (Yamamoto et al. 2004).

Another noticeable pattern is the relatively recent (0.24–0.64 Mya, in the middle Pleistocene) penetration of TH4 from the Pacific side to the Sea of Japan side of Honshu Island, across the Central Highlands region (around the Fossa Magna). The Central Highlands region is the second most important boundary for freshwater fish in the Japanese Archipelago (Nishimura 1974; Watanabe 2012), and its uplift in the Plio-Pleistocene has caused deep differentiation in many fish groups at interand intraspecific levels (e.g., bitterlings, minnows, loaches, and catfishes; see Watanabe et al. 2016). The expansion of the range of *T. hakonensis* across this mountainous area may have been enabled by its ability to inhabit the upper reaches of rivers (e.g., Nakagawa 2014), providing more opportunities to expand their range via river captures across watersheds in mountainous areas (e.g., Kitagawa et al. 2001; BurrIDGE et al. 2006). Finer-scale analyses with highly sensitive molecular markers for *T. hakonensis* will contribute to a deeper understanding of the relationship between ecology and distribution in freshwater fishes.

**Evolutionary history of *Tribolodon*.** Our analyses suggest that *Tribolodon* + *Pseudaspius* species diversified from the Late Miocene to the early Pleistocene, emphasizing the importance of the environmental changes in the Sea of Japan during those periods in the formation of diadromous freshwater fish fauna in this area. Some cold water-adapted euryhaline fishes, such as sticklebacks (*Gasterosteus* and *Pungitius*) and sculpins (*Cottus*), have also been inferred to have diversified around the Sea of Japan in those periods (Higuchi and Goto 1996; Yokoyama and Goto 2005; Kitano et al. 2007; Takahashi et al. 2016). The Sea of Japan opened from about 20 to 14 Mya (e.g., Jolivet et al. 1994; Tada 1994; Yonekura et al. 2001; Kitamura 2010), when the common ancestor of *Tribolodon* and *Pseudaspius*, or that of



Far Eastern phoxinins, probably inhabited this area (Fig. 4). Salinity and temperature in the Sea of Japan fluctuated with climatic changes and the opening and closing of its southern strait (the Tsushima Strait) (e.g., Iijima and Tada 1990; Kitamura and Kimoto 2006). These conditions may have facilitated the diversification of freshwater fishes involving evolutionary changes associated with the diadromous lifestyle.

The phylogenetic relationships and divergence times of the *Tribolodon* + *Pseudaspius* species remain uncertain, although these elements are essential to reconstruct the evolution of diadromy in this group. Our results, based on partial mtDNA data, do not allow us to propose a strong phylogenetic hypothesis for *Tribolodon* + *Pseudaspius* species. However, the topology of our BI tree (Fig. 4) is identical to that produced by complete mitochondrial genome data (Imoto et al. 2013). This mtDNA relationship does not support the monophyly of diadromous *Tribolodon* species (i.e., *T. brandtii* and *T. hakonensis*). However, allozyme data support different tree topologies, i.e., with *Pseudaspius leptocephalus* positioned outside of *Tribolodon* (Kartavtsev et al. 2002; Sakai et al. 2002) and with the diadromous species, *T. brandtii* and *T. hakonensis*, being monophyletic (Sakai et al. 2002). Interspecific hybridization (Sakai and Hamada 1985; Sakai 1995) and allelic introgression (Sakai et al. 2002) have also been reported among *Tribolodon* species; these findings are supported by a small degree of interspecific mtDNA introgression found in our preliminary analyses (unpublished data). These aspects may introduce bias into the matrilineal genealogy for interspecific relationships.

Different studies have also introduced discrepancies among divergence times; our estimations were much smaller (e.g., about 6–12 Myr for tMRCA of *Tribolodon* + *Pseudaspius*) than those proposed by Imoto et al. (2013) (13–23 Myr, as measured from figure 5 of Imoto et al. 2013). This seems to be due to differences in the calibration data used for dating the tree. We used a geographic event and the molecular evolutionary rates obtained from cyprinids and other teleosts as time constraints. Although our two constraints were roughly concordant with each other, their applicability might need to be reexamined. On the



other hand, the estimate of Imoto et al. (2013) should also be treated with caution, because their tree was calibrated with nodes 10 to a 100 times older (about 60–140 Myr). Different datasets from multiple nuclear genes and a more inclusive approach to time calibration are necessary to more accurately estimate divergence times in future research.

**Acknowledgments** We thank S. Amano, A. Goto, R. Kakioka, H. Kato, K. Katsura, H. Kohno, K. Iguchi, S.-R. Jeon, T. Kaneko, S. N. Safronov, H. Sugiyama, R. Tabata, Y. Takahashi, T. Tokuhara, V. V. Tsuiger, G. Yamamoto, Y. Yamazaki, and A. Zama for their help in the collection of specimens. This study was partially supported by the Sumitomo Foundation and JSPS KAKENHI (No. 06041004, 09041138, 21370035, 26291079, 26250044, and 17H03720).

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## Figure legends

**Fig. 1** Distribution ranges of four *Tribolodon* species with two sub-species (shaded area). “?” denotes area lacking information. Drawn after Sakai (1995) and Brykov et al. (2013)

**Fig. 2** Sampling localities of four *Tribolodon* species. *Closed squares*, *T. brandtii*; *open circles*, *T. sachalinensis*; *triangles*, *T. nakamurai*; *closed circles*, *T. hakonensis*

**Fig. 3** ML tree of *Tribolodon* and closely related species based on partial *cytb* sequences (758 bp; “short sequences”). Intraspecific haplotype groups are labeled, e.g., *TBB1*. *Numbers at internodes* represent bootstrap probability values ( $\geq 70\%$ ) for 1,000 replicates

**Fig. 4** Bayesian time-calibrated tree of *Tribolodon* and closely related species based on partial mtDNA sequences (2,941 bp from *cytb*, COI, and 16S; “long sequences”). Intraspecific haplotype groups are labeled, e.g., *TBB1*. *Numbers at internodes* represent Bayesian posterior probability ( $\geq 0.9$ )/ML bootstrap probability ( $\geq 70\%$ ). *Nodes with circled numbers* are those referred to in the text. *Node 8 (\*)* is the node at which the geographic calibration was applied (see “Materials and methods”)

**Fig. 5** Haplotype network of *Tribolodon brandtii* with a distribution map of haplotype groups. *Abbreviations of haplotype groups* correspond to those of Figs. 3 and 4. Haplotype groups are represented by *different symbols* on the map. *Broken lines* indicate the boundaries of distribution of haplotype groups. *Shaded areas* represent the distribution of the species. *The sizes of circles* in networks reflect the relative frequency of haplotypes. Only major haplotypes are labeled in the network. *Black dots* represent missing haplotypes not detected among our samples

**Fig. 6** Haplotype network of *Tribolodon nakamurai* with distribution map. *Shaded areas* represent the distribution of the species. *The sizes of circles* in networks reflect the relative

frequency of haplotypes. *Black dots* represent missing haplotypes not detected among our samples

**Fig. 7** Haplotype network of *Tribolodon sachalinensis* with distribution map. *Shaded areas* represent the distribution of the species. *The sizes of circles* in networks reflect the relative frequency of haplotypes. Only major haplotypes are labeled in the network. *Black dots* represent missing haplotypes not detected among our samples. The localities of haplotypes showing differentiation from major haplotypes with several missing haplotypes (sa10, 17) are indicated by *symbols* on the map

**Fig. 8** Haplotype network of *Tribolodon hakonensis* with distribution map. *Abbreviations of haplotype groups* correspond to those of Figs. 3 and 4. Networks and distribution maps represent one or two haplotype groups (**a–d**). *Broken lines* indicate the distribution boundaries of the haplotype groups. *Shaded areas* represent the distribution of the species. *The sizes of circles* in networks reflect the relative frequency of different haplotypes. Only major haplotypes are labeled in the network. *Black dots* represent missing haplotypes not detected among our samples. *Arrowhead* in the network of each group indicates the root position in the overall network (**e**). *Arrows* in maps indicate the direction of range expansion that was inferred from the tip and interior relationship of haplotypes in the network

**Table 1** Localities, sample size (*n*), and haplotype frequencies of four species of *Tribolodon* and comparative species

Code	River system	Locality	<i>n</i>	Group ( <i>n</i> <sup>1</sup> )	Haplotype ( <i>n</i> <sup>2</sup> )	GEDIMAP ID
<i>Tribolodon brandtii brandtii</i>						
4	Mukawa River	Hokkaido	2	TBB1	bb07, bb09	P2201
9	Omono R.	Akita	2	TBB1	bb02(2)	P2202
14	Shinano R.	Niigata	5	TBB1	bb01, bb02, bb06(2), bb08*	P2203
16	Oyabe R.	Toyama	9	TBB1	bb01*, bb03*(6), bb04, bb05	P2204
44	Busse Lagoon	Sakhalin, Russia	4	TBB1	bb06*, bb08(3)	P2205
47	Peter the Great Bay	Primorsky, Russia	2	TBB2	bb10, bb11*	P2206
49	Songchon R.	Korea	5	TBB2	bb10, bb11(4)	P2207
<i>T. brandtii maruta</i>						
18	Ofunato Bay	Iwate	5	TBM	bm01, bm02(4)*	P2208
20	Kasumigaura Lake	Ibaraki	4	TBM	bm01(3), bm03*	P2209
21	Tama R.	Tokyo	1	TBM	bm01	P2210
22	Tokyo Bay	Tokyo	5	TBM	bm01(5)	P2211
<i>T. sachalinensis</i>						
1	Teshio R.	Hokkaido	8	TS	sa01*, sa03, sa04, sa07, sa08, sa12*, sa15*(2)	P2212
2	Tokoro R.	Hokkaido	5	TS	sa17*(5)	P2213
3	Ishikari R.	Hokkaido	9	TS	sa02, sa06*, sa10, sa11, sa13*(3), sa14, sa16	P2214
4	Mukawa River	Hokkaido	3	TS	sa01(3)	P2215
6	Tanabu River	Aomori	18	TS	sa01(18)	P2216
12	Mogami River	Yamagata	3	TS	sa05(3)	P2217
45	Lytutoga R.	Sakhalin, Russia	3	TS	sa04, sa9*, sa17	P2218
46	Tumnin R.	Primorsky, Russia	8	TS	sa06(8)	P2219
<i>T. nakamura</i>						
10	Koyoshi R.	Akita	1	TN	n01	P2220
12	Mogami R.	Yamagata	4 <sup>3</sup>	TN	n02*(3), n03	P2221
13	Agano R.	Niigata	2 <sup>3</sup>	TN	n01, n02	P2222
<i>T. hakonensis</i>						
1	Teshio R.	Hokkaido	2	TH1(1), TH3(1)	h04*, h37	P2223
2	Tokoro R.	Hokkaido	2	TH1	h01*(2)	P2224
3	Ishikari R.	Hokkaido	1	TH3	h51	P2225
5	Ohno R.	Hokkaido	2	TH1(1), TH3(1)	h03, h42*	P2226
7	Ohata R.	Aomori	10	TH3	h37, h42, h46, h52(7)	P2227
8	Osorezan L.	Aomori	11	TH3	h47(8), h49, h50(2)	P2228
11	Nikko R.	Yamagata	9	TH3(7), TH4(2)	h37(2), h39*, h42(3), h43, h60*, h61	P2229
15	Chikuma R.	Nagano	12	TH3(9), TH4(3)	h37(2), h39, h42(4), h44, h45, h60(2), h62	P2230
17	Kozuchi R.	Iwate	7	TH2(3), TH3(4)	h06*(2), h08*, h37*(4)	P2231
18	Ofunato Bay	Iwate	10	TH3	h37(7), h38(2), h52	P2232
19	Kinu R.	Tochigi	6	TH2	h06(4), h07, h08	P2233
21	Tama R.	Tokyo	1	TH4	h54	P2234
23	Kaname R.	Kanagawa	9	TH2	h06(8), h09	P2235
24	Tenryu R.	Shizuoka	3	TH4	h54(2), h62*	P2236
25	Toyo R.	Aichi	9	TH4	h58(9)	P2237
26	Kiso R.	Gifu	10	TH4	h53*, h54(6), h58(2), h59*	P2238
27	Miya R.	Mie	4	TH4	h54(3), h55	P2239
28	Koza R.	Wakayama	4	TH4	h54, h56*(2), h57	P2240
29	Ado R.	Shiga	20	TH5	h10(2), h11, H12(7), h33(4), h34(4), h35(2)	P2241
30	Lake Biwa	Shiga	16	TH5	h10*(7), h12(4), h33*(4), h34	P2242
31	Kita R.	Fukui	20	TH5	h27*, h28(11), h29(6), h30(2)	P2243
32	Minami R.	Fukui	20	TH5	h28(17), h29(3)	P2244
33	Ibo R.	Hyogo	10	TH5	h28(2), h32(4), h36(4)	P2245
34	Takahashi R.	Okayama	8	TH5	h19(4), h24(4)	P2246
35	Kando R.	Shimane	7	TH5	h24(6), h31	P2247
36	Kotou R.	Yamaguchi	6	TH5	h19(4), h20(2)	P2248
37	Awano Harbor	Yamaguchi	9	TH5	h19(6), h24(3)	P2249

38	Shimanto R.	Kochi	10	TH5	h13*(2), h14(2), h15, h16, h17, h18*(3)	P2250
39	Oita R.	Oita	5	TH5	h19(4), h21	P2251
40	Matsuura R.	Saga	11	TH6	h66*(10), h67	P2252
41	Chikugo R.	Fukuoka	1	TH5	h23*	P2253
42	Sendai R.	Kagoshima	10	TH5	h22, h25, h26(8)	P2254
43	Sago R.	Tsushima Island, Nagasaki	10	TH6	h68(10)	P2255
44	Busse Lagoon	Sakhalin, Russia	3	TH1(1), TH3(2)	h05, h40, h48	P2256
45	Lyutoga R.	Sakhalin, Russia	1	TH3	h41*	P2257
46	Tumnin R.	Primorsky, Russia	8	TH1(3), TH3(5)	h01, h02(2), h37(2), h47(3)	P2258
47	Peter the Great Bay	Primorsky, Russia	2	TH6	h64(2)	P2259
48	Samchokoship	Korea	3	TH6	h63*, h64, h65	P2260
<i>Pseudaspius leptcephalus</i>						
	Chita R., Amur R. basin	Chita, Russia	1			
<i>Rhynchocypris lagowskii steindachneri</i>						
	Ishida R.	Shiga	1			
<i>Rhynchocypris oxycephalus</i>						
	Ishida R.	Shiga	1			

Locality codes correspond to those of Fig. 1

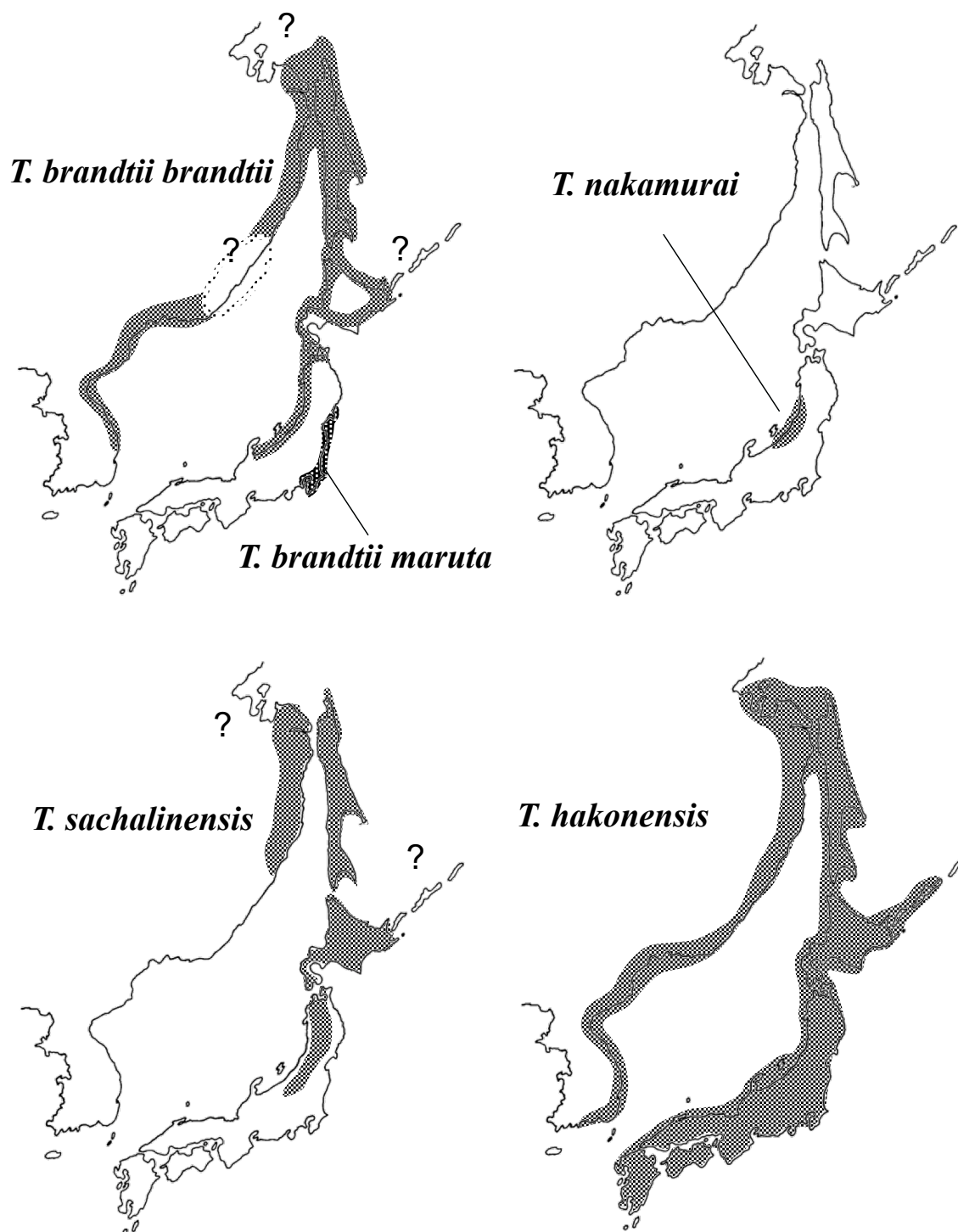
Group names correspond to those of Figs. 3, 4, 5, 6, 7, 8

<sup>1</sup>Number of specimens when two or more groups coexisted

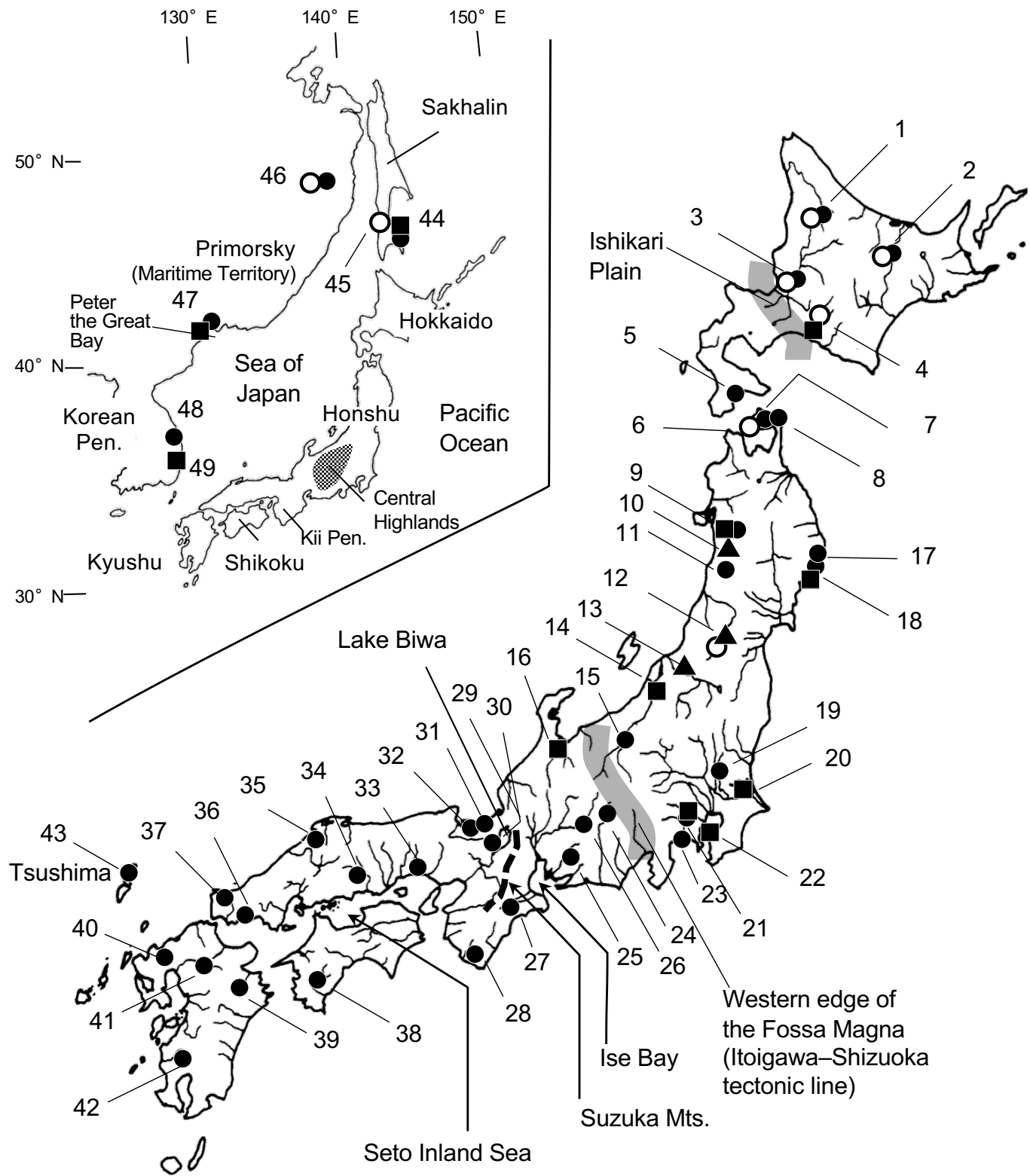
<sup>2</sup>Number of specimens (only shown for haplotypes with  $\geq 2$ )

<sup>3</sup>Including sequences from database

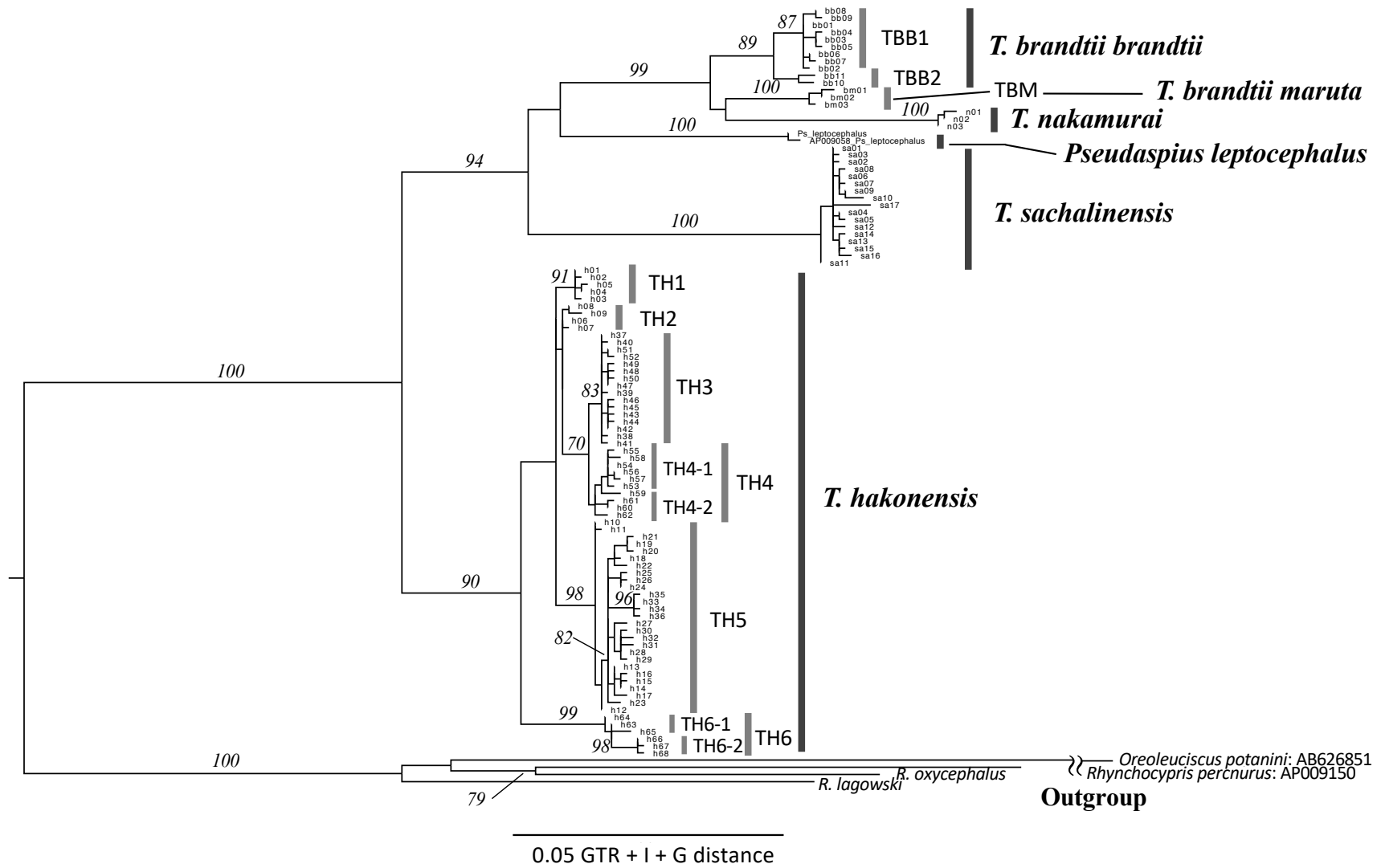
\*Haplotypes for which longer sequences were obtained



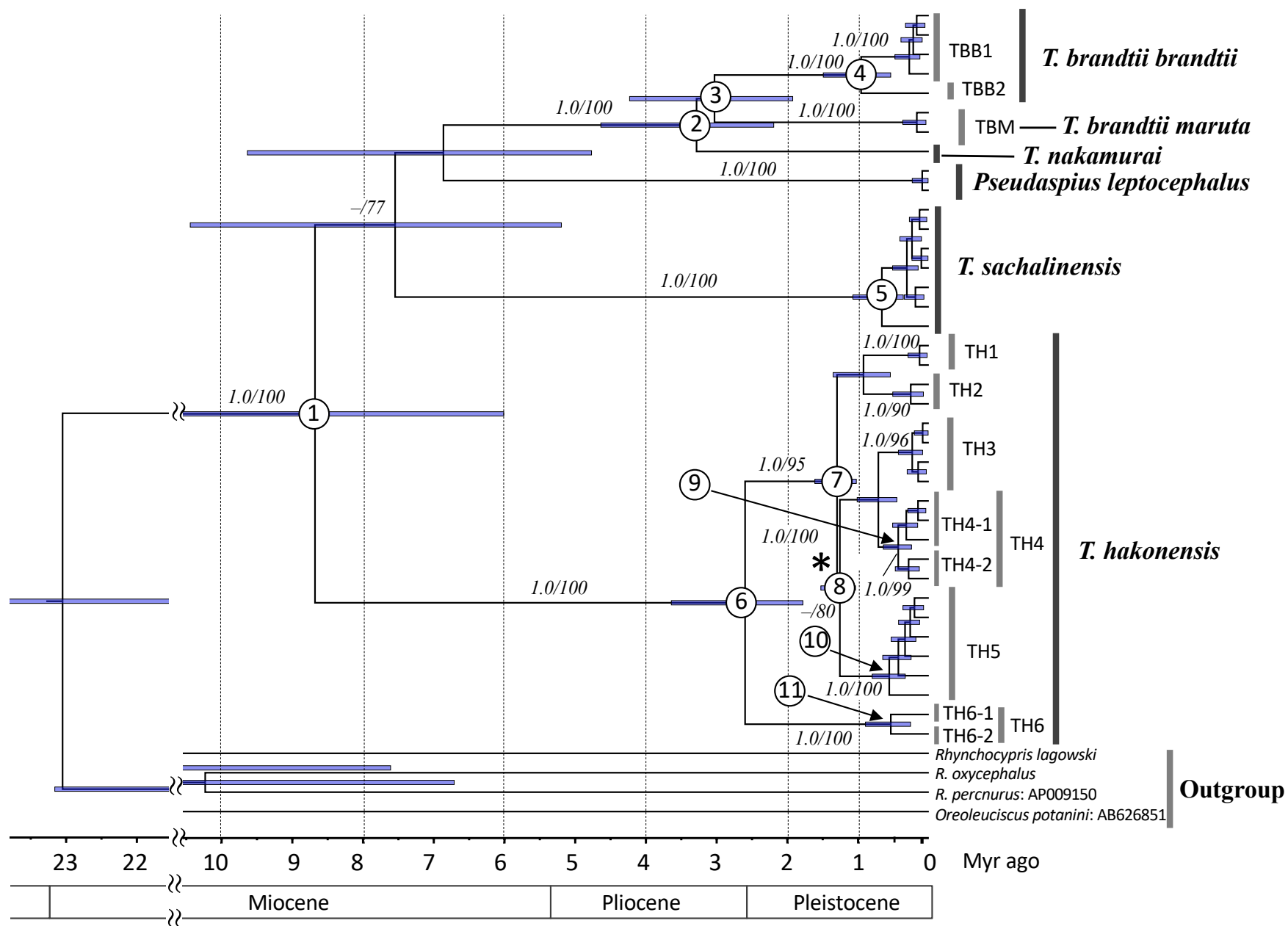
↑Fig.1



↑Fig.2



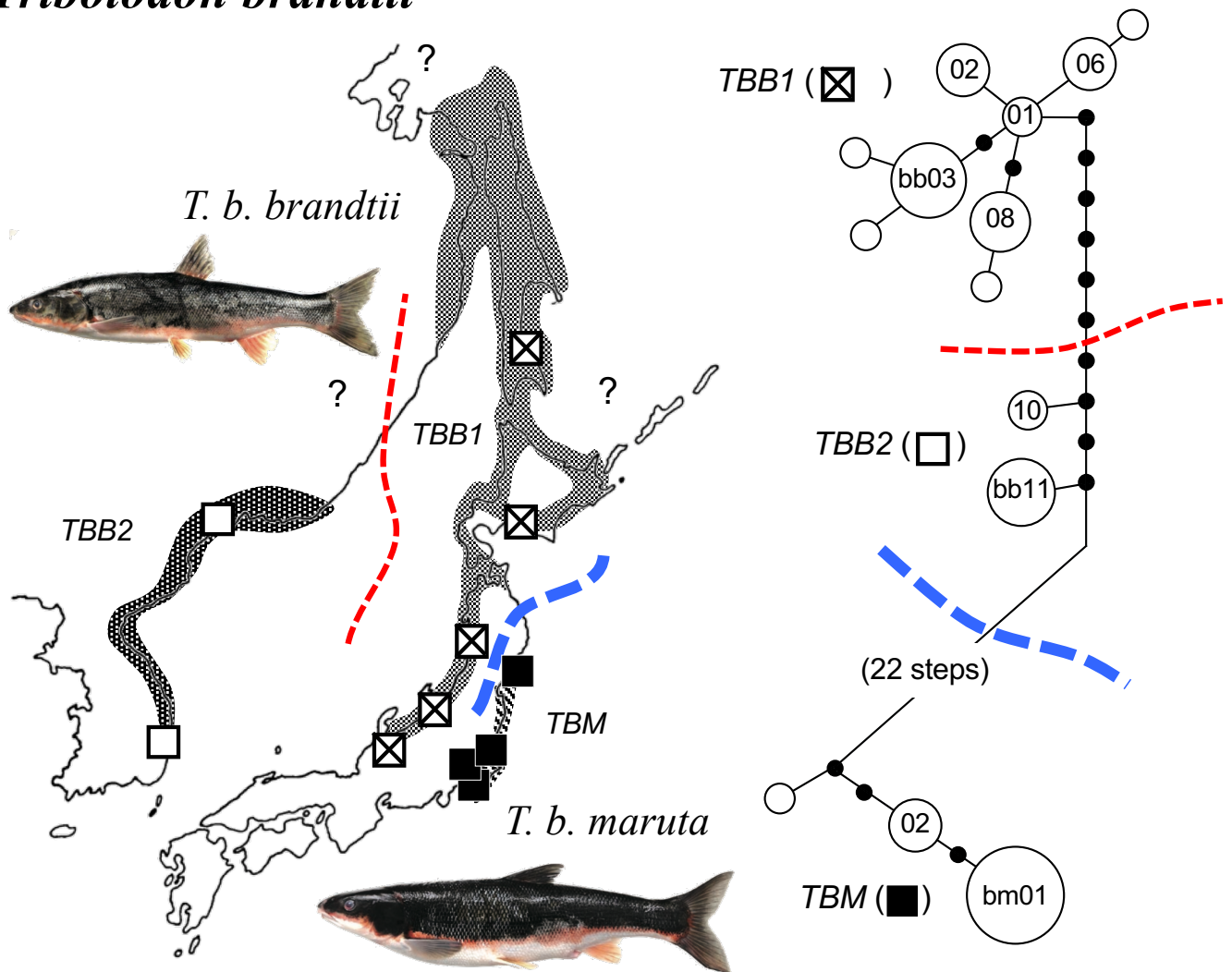
↑Fig.3

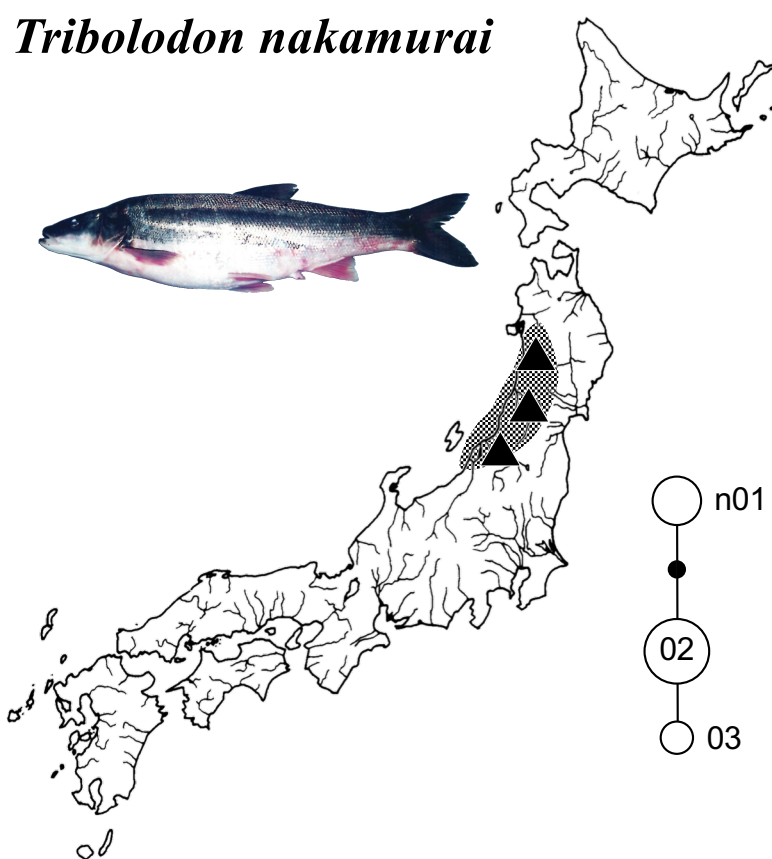


↑Fig.4

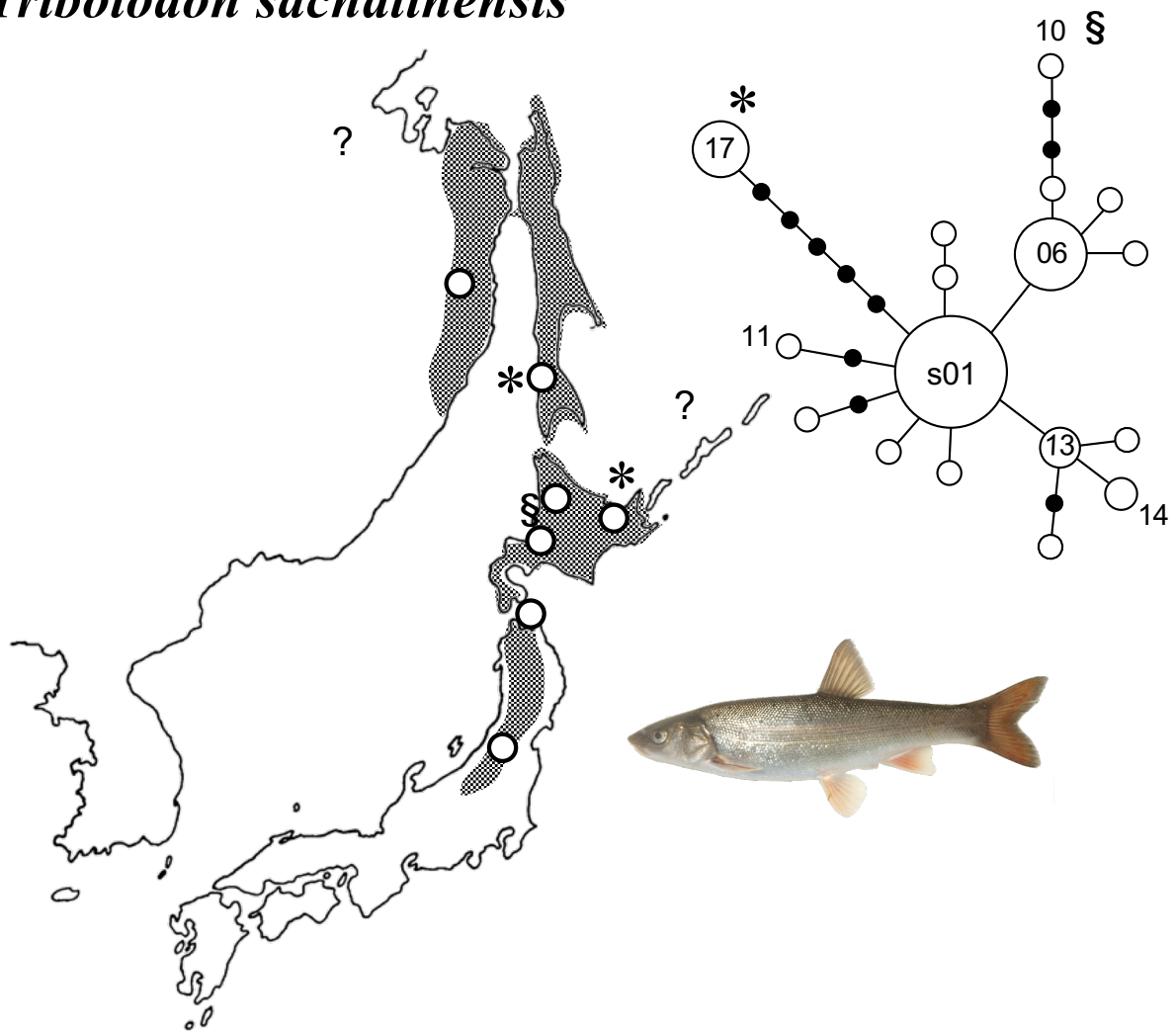


# *Tribolodon brandtii*

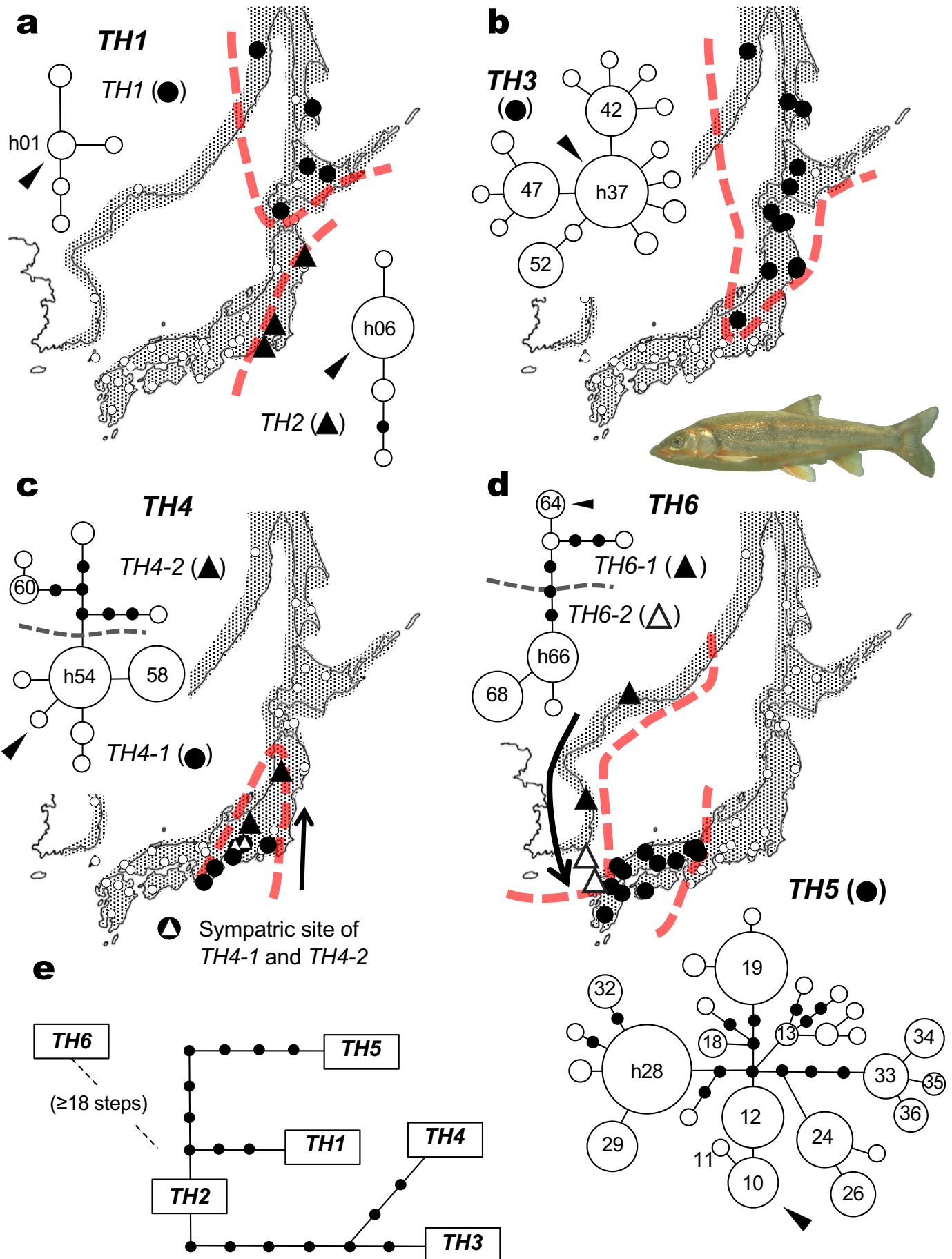




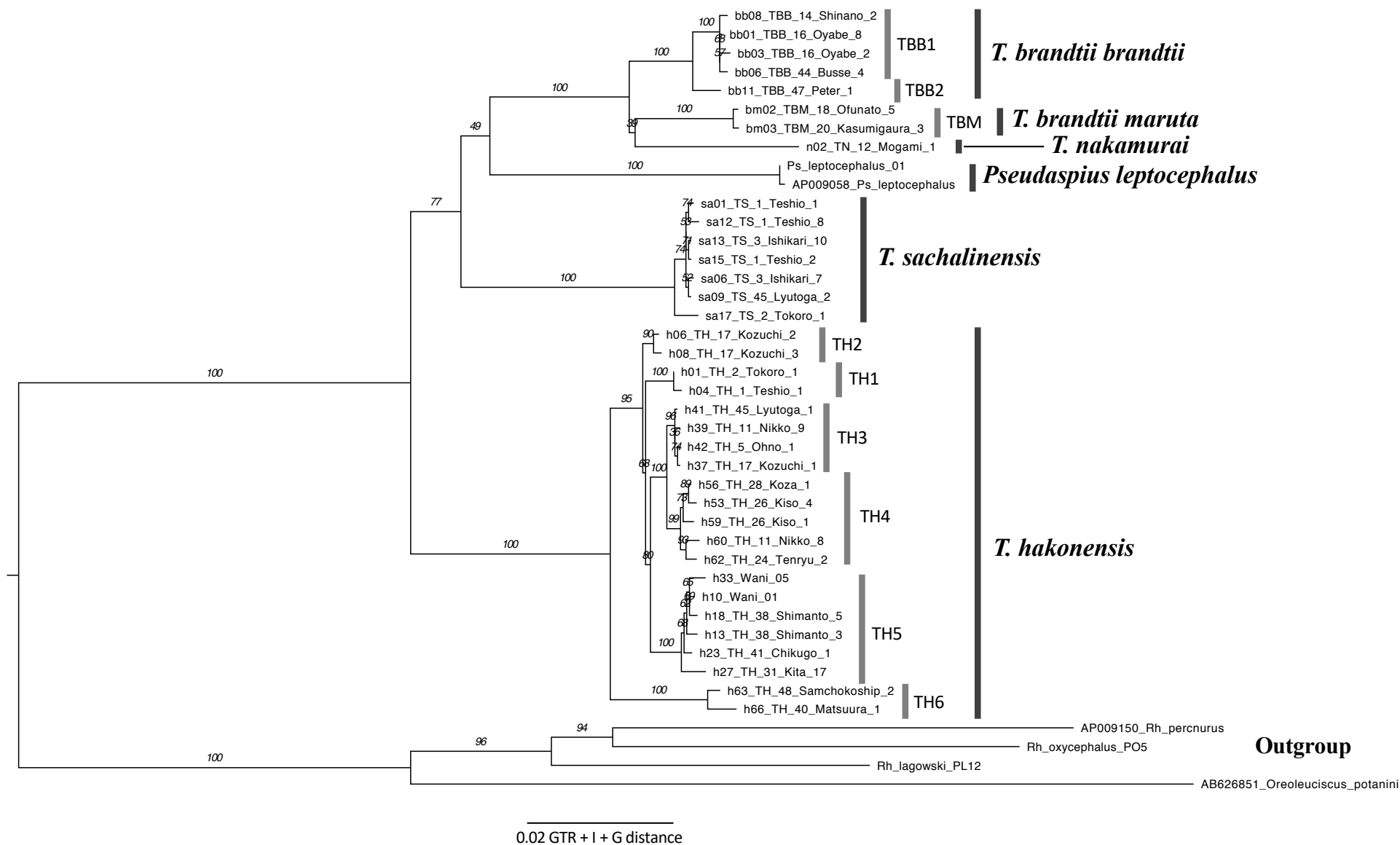
# *Tribolodon sachalinensis*



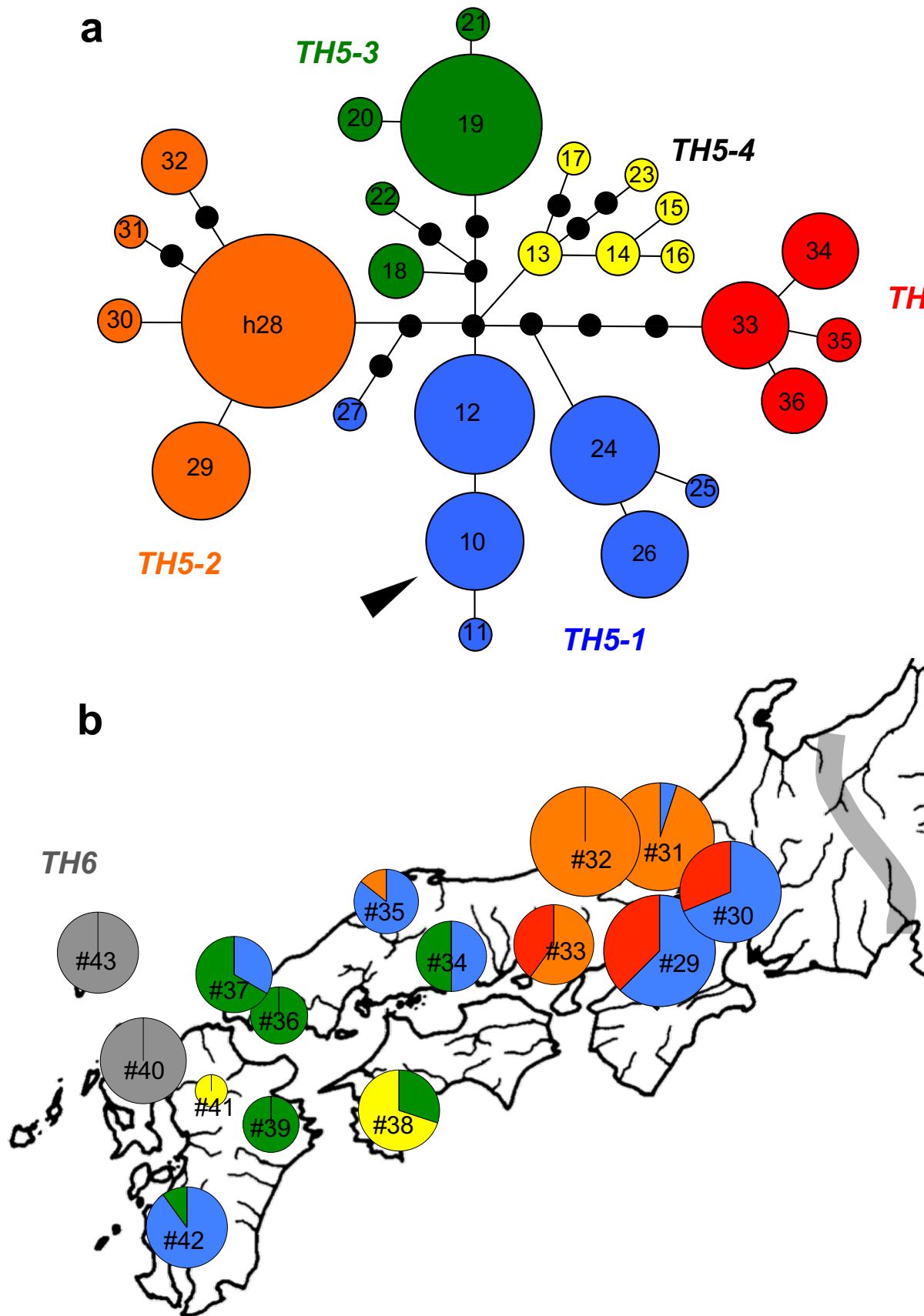
# *Tribolodon hakonensis*



↑Fig. 8

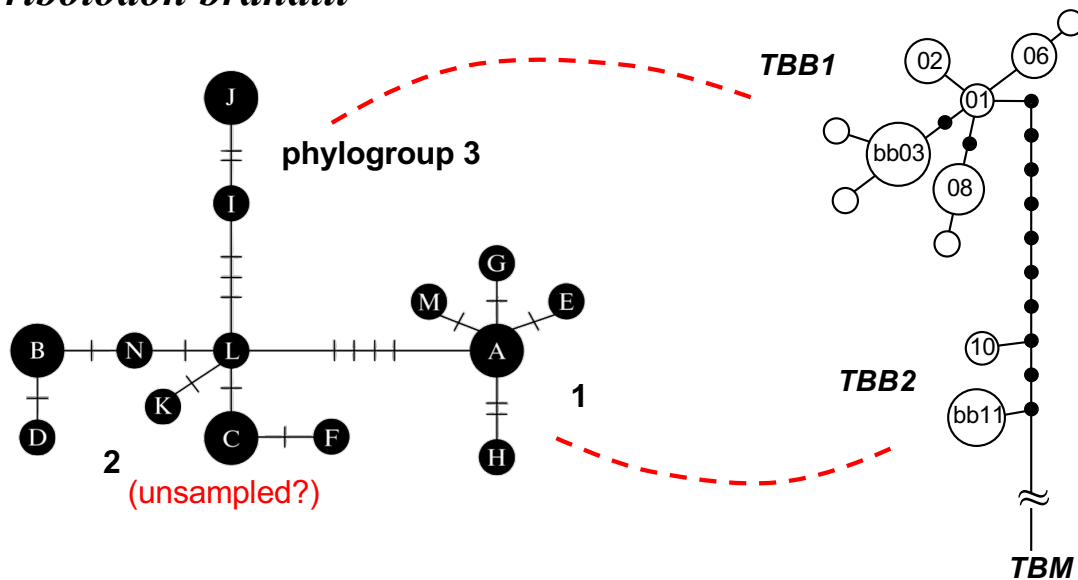


**ESM Fig. S1** ML tree of *Tribolodon* and closely related species based on partial mtDNA sequences (2,941 bp from cytb, COI, and 16S; “long sequences”). *OTU labels* denote the haplotype name, species/subspecies code, locality code, and specimen ID. *Numbers at internodes* denote bootstrap probability values for 1,000 replicates

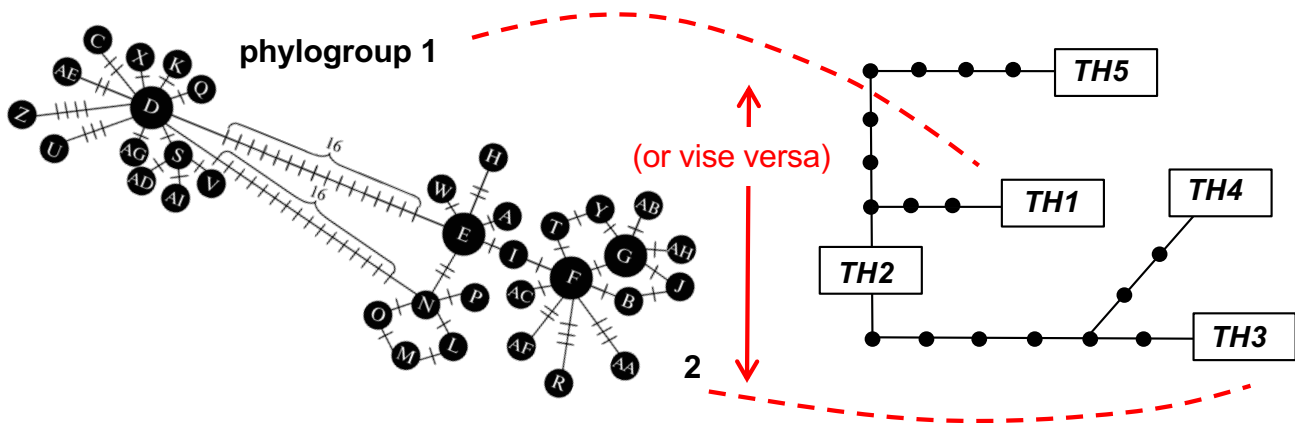


**ESM Fig. S2** Subgroups of the haplotype group TH5 of *Tribolodon hakonensis* (a) and distribution map (b). Grouping was conducted by the nesting rule (Templeton and Sing 1993), and 2-step clades (TH5-1–TH5-5) are indicated by different colors. *Arrowhead* in the network indicates the position of the root based on the overall network of *T. hakonensis* (see Fig. 8e). Haplotype frequencies in local samples (denoted with #) are expressed by *pie charts*

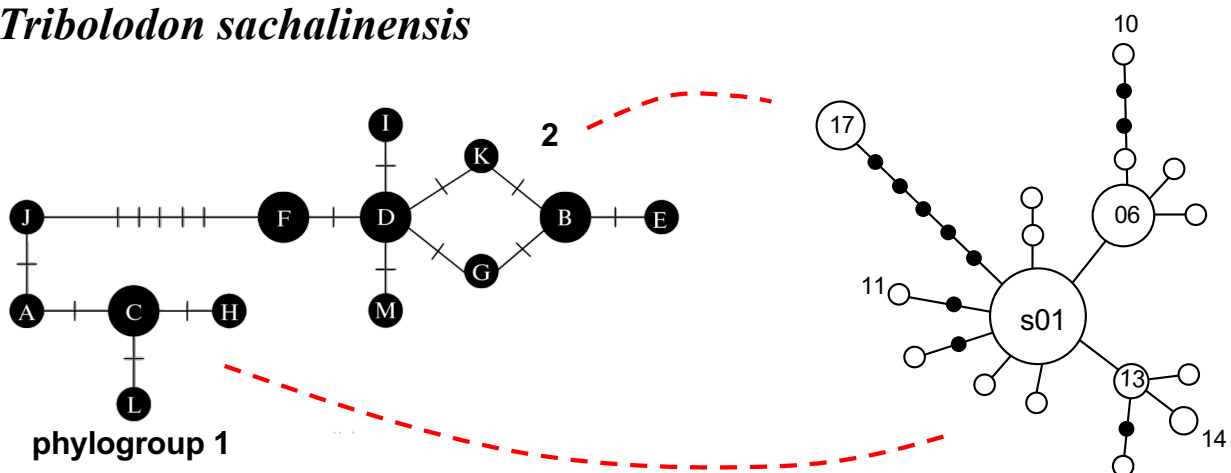
**a** *Tribolodon brandtii*



**b** *Tribolodon hakonensis*



**c** *Tribolodon sachalinensis*



**ESM Fig. S3** Possible correspondence (red broken lines) between the subgroups in the haplotype networks based on RFLP data in Brykov et al. (2013) (*left*) and those of *cytb* sequences by the present study (*right*), inferred from the distribution patterns and degrees of divergence. Numbers of hatches in connecting branches in *left panels* correspond to number of the nucleotide substitutions between haplotypes. See Figs. 5, 7 and 8 for the detailed explanation for the networks in *right panels*